APPENDIX C: DECLARATION OF DR. STEPHEN JOHNSTON

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Stephen Albert Johnston Michael A. Barry Wayne C. Lai	§ § § § §	 Examiner: C. Low
Serial No.: 08/421,155	§ §	Group Art Unit: 1804
Filed: April 7, 1995	§ §	 Atty. Dkt. No.: UTSD:411/KIT
For: EXPRESSION LIBRARY IMMUNIZATION	§ §	

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on the date below:

June 20, 1997 — Barbara S. Kitchell
Date — Barbara S. Kitchell

DECLARATION OF STEPHEN A. JOHNSTON

I, STEPHEN A. JOHNSTON HEREBY DECLARE AS FOLLOWS:

1. I, along with Michael Barry and Wayne C. Lai, am a co-inventor of the subject matter disclosed and claimed in the referenced patent application.

2. Attachment 1 to this declaration is a summary of reasons other scientists, including myself, did not think expression library immunization would be feasible.

3. Attachment 2 to this declaration is a commentary published by Jeffrey B. Ulmer and Margaret A. Liu. Even after disclosure of initial success with ELI, their comments indicate residual doubt as to whether the method would work.

4. I have underlined selected portions of several publications in the Exhibits (E, G, H, I and J) to support what I believe to be relevant to the invention.

5. All statements made in this Declaration on information and belief are believed to be true, and these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and may jeopardize the validity of this application or any patent issuing thereon.

6/20/97
Date

Stephen A. Johnston

REASONS PEOPLE THOUGHT <u>ELI</u> WOULD <u>NOT</u> WORK

1. Bacterial Genes would not sufficiently express in mammalian cells.

2. The level of expression would be too low to elicit an immune response.

3. ELI would only work in natural pathogen/host systems.

4. Interference and/or Dominance would prevent expression of valuable antigens in mixtures.

5. Tolerance would be produced by low level expression of pathogen genes.

1. **BACTERIAL GENES WOULD NOT SUFFICIENTLY EXPRESS IN MAMMALIAN CELLS.**

- The Nature, 1995 paper on ELI was the first demonstration of genetic immunization against a bacterial pathogen. Only viral genes and Plasmodium (malaria, eukaryote) genes had been used.

- No one knew if bacterial reading frames would express well enough in mammalian cells.

- Viral and tumor genes use mammalian transcription and translation signals as do eukaryotic pathogens (fungi and parasites).

- Bacterial have divergent codon usage. Mycoplasma pulmonis, the model we used, reads mammalian Trp codon as a Stop codon.

- By using a bacteria, particularly *M. pulmonis* ELI subjected to its most rigorous test.

2. THE LEVEL OF EXPRESSION WOULD BE TOO LOW TO ELICIT AN IMMUNE RESPONSE.

- At the time of filing and even at the time of the Nature, 1995 publication the lowest amount of DNA used successfully for genetic imm. was 400 ng for muscle injection (Fynan, *et al.*, 1993) and 80 ng for gene gun (Eisenbraun, *et al.*, 1993). These examples used optimized expression of viral or human genes - not bacterial.

- At these sensitivities one would have predicted a maximum of 200 viral genes could be screened at once. With ELI, protection was demonstrated with up to 27,000 bacterial genes at once.

- This represents a sensitivity of 0.4 ng at least 320 times more sensitive than previous reports.

3. ELI WOULD ONLY WORK IN NATURAL PATHOGEN/HOST SYSTEMS.

- *Mycoplasma pulmonis* is natural pathogen of mice. Will this system work in model systems which would be necessary for screening vaccine candidates.

- ELI has been demonstrated using mice in model systems for *Mycobacterium tuberculosis* (human pathogen) and *Chlamydia psittaci* (cattle pathogen).

INTERFERENCE AND/OR DOMINANCE WOULD PREVENT EXPRESSION OF VALUABLE ANTIGENS IN MIXTURES.

- Introduction of two or more proteins often leads to immune response to only one. See *Ulmer* and *Liu*, 1995 and references therein.

- Most immunologists thought there would be too much dominance or interference to screen complex mixtures.

- At the time of the Nature, 1995 paper on ELI, no one had reported the introduction of more than one gene at once for protection.

- To date, there is only one report (Dolan, *et al.*, 1996, J. Expt. Med. 183:1739-46) of introducing two genes to confer protection (malaria model). These were defined genes.

- In contrast to predictions of interference, the ELI method demonstrates that fragmentation of genes in a library gives improved response. See data on fragmentation of HIV Gag gene as cited. THIS WAS THE OPPOSITE OF THAT PREDICTED BY ULMER AND LIU AND GENERALLY HELD IN THE FIELD.

5. TOLERANCE WOULD BE PRODUCED BY LOW LEVEL EXPRESSION OF PATHOGEN GENES.

- It is well-established in immunology that low level expression of a foreign antigen can "tolerize" the animal to subsequent exposure. If this happened ELI would actually enhance the disease rather than protect.
- The letter from Dr. Lyons expands on this potential problem.
- In fact, in the ELI screening of the *M. tuberculosis* libraries we found 3 of 4 libraries that apparently tolerized to infection.

ELI's cloning: expression library immunization and vaccine antigen discovery

Jeffrey B. Ulmer and Margaret A. Liu

munity in an animal model. This suggests that further refinement of this approach might be used to delineate the protective antigens of a pathogen.

The idea that plasmid DNA might be used to express proteins *in situ*, leading to the generation of immune responses against the expressed protein, was suggested by the demonstration that intramuscular injection of plasmids encoding reporter genes results in reporter-protein synthesis *in situ*[3]. This was extended by the successful use of particle bombardment with gold particles coated with DNA plasmids encoding human growth hormone to transfect dermal and epidermal cells. The hormone was secreted by host cells and subsequently induced antibody responses[4]. The utility of DNA as a vaccine was first demon- infection (for example, for hepatitis δ virus[8]).

One of the most daunting tasks in developing a subunit vaccine is the identification of the relevant protective antigens of a pathogen. This is not an impediment for some viruses, such as influenza, which have a small number of antigens, but is particularly problematic for other viruses, parasites and bacteria that have large genomes and express many proteins. Traditional approaches dictate that purified or partially purified protein preparations should be tested as subunit vaccines.

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for inoculation. Barry *et al.* found that two separate *M. pulmonis* sibs, each containing approximately 3000 members, induced protective immunity in a mouse model of disease. Sequential partitioning of these sibs to smaller subsets of the genome could lead to the identification of the protective antigens of *M. pulmonis*. This technique might also apply to the discovery of vaccine antigens against other pathogens.

For the moment, however, enthusiasm should be tempered because of the potential caveats inherent in this technique. The simultaneous expression of many antigens could lead to antigenic competition, in which normally immunogenic proteins might not induce immune responses in the presence of other immunodominant antigens that direct the energies of the immune

① Competiti

COMMENT

system to nonproductive responses. For example, some antigens are known to be the focus of immune responses[9], while others can induce immunological nonresponsiveness[10].

Expression of partial proteins (which will occur for many open reading frames because DNA fragments are used in preparing the sibs) could result in the loss of conformational epitopes. This may be more of a problem for neutralizing antibody responses, but improper folding and inappropriate intermolecular interactions or intracellular trafficking of these partial proteins might also occur, and might prevent processing and presentation by major histocompatibility complex molecules. These potential problems may be more significant for bacterial and parasitic proteins that are not normally expressed in eukaryotic cells.

② Conformation

③ Interference

It is not known if simultaneous expression of several thousand proteins *in vivo* can be achieved by DNA vaccination. The two com-...s of DNA admini-

④ Expression Level

Despite these potential limitations, many of which may prove to be inconsequential or may be overcome by future developments, ELI may become a useful tool in the often rate-determining and impractical process of vaccine antigen discovery.

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Response from Johnston, Barry and Lai

We agree with Jeffrey Ulmer and Margaret Liu that enthusiasm for expression library immunization (ELI) technology should be tempered by the potential problems

ally takes higher antigen expression to raise antibodies than to elicit cellular responses. (2) Entities with very large genome sizes (for example, *Plasmodium* or cancer cells) will be more difficult to tackle in direct proportion to the size of the genome. (3) The model system is criti-